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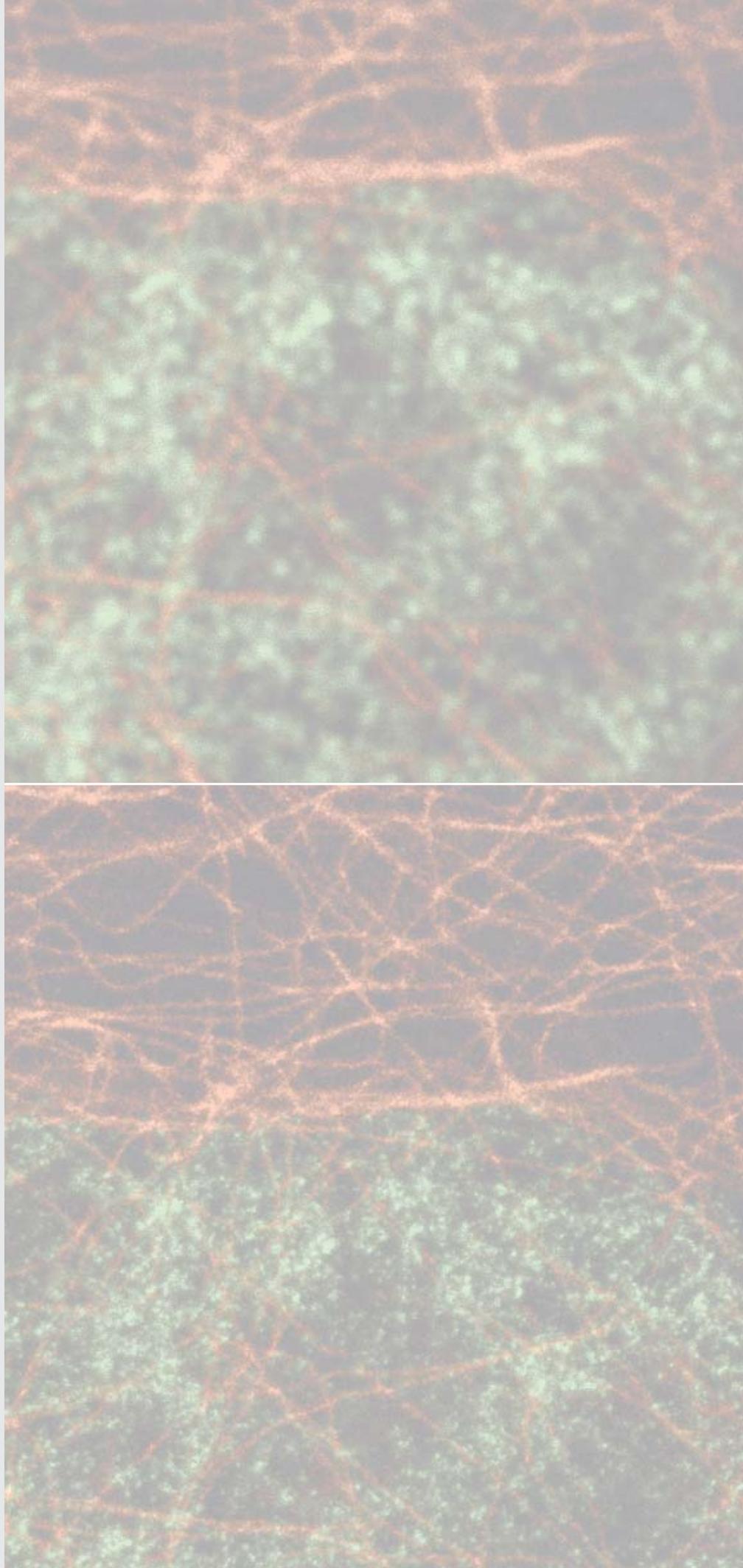
Dual Color STED Imaging
Principles and Tips & Tricks

Leica
MICROSYSTEMS

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Cover:
HeLa Cells
Red: Tubulin, Chromeo 494
Green: Histone H3, ATTO 647N



Stimulated Emission Depletion Microscopy – Basics

Great insights have been obtained from fluorescence microscopy, but the study of subcellular architecture and dynamics can be lost in a blur due to resolution limited by diffraction. Fortunately, several approaches to overcome this limitation have been developed over the last decades¹.

The concept of STimulated Emission Depletion (STED) microscopy, which shrinks the effective focal spot to circumvent the Abbe limit, was first described by Stefan Hell in 1994². Currently, the Leica TCS STED and the Leica TCS STED CW employ this technology to provide fast and easy access to super-resolution.

The basic principle is simple: As in confocal microscopy, a focal spot is scanned over the specimen, and the fluorescence signal emitted is registered as pixels to build an image. The “trick” to achieve super-resolution is to control the area inside the focal spot from where fluorescence can be emitted. To this end two super-imposed lasers (an excitation and a depletion beam) are applied. The super-resolution trick is implemented by modifying the depletion beam with a phase plate to generate a doughnut shaped area in the focal plane: the STED PSF

(Fig. 1). The fluorophores within the area of the depletion beam will be forced back to the ground state. The arrangement of excitation and depletion beams achieves an area of emitted fluorescence that is smaller than the diffraction limit.

The resolution of a STED microscope is given by

$$\Delta x \approx \frac{\lambda}{2NA\sqrt{1 + \frac{I}{I_s}}}$$

where NA is the numerical aperture and λ is the STED wavelength. I is the intensity of the depletion laser, and I_s is a dye and the depletion wavelength specific parameter.

Theoretically, resolution can be infinitely increased. In practice, it depends on the intensity of the depletion laser (I) and on the dye specific parameters, I_s .

Fig. 2 gives details on the photo physics inside the focal spot (see page 4).

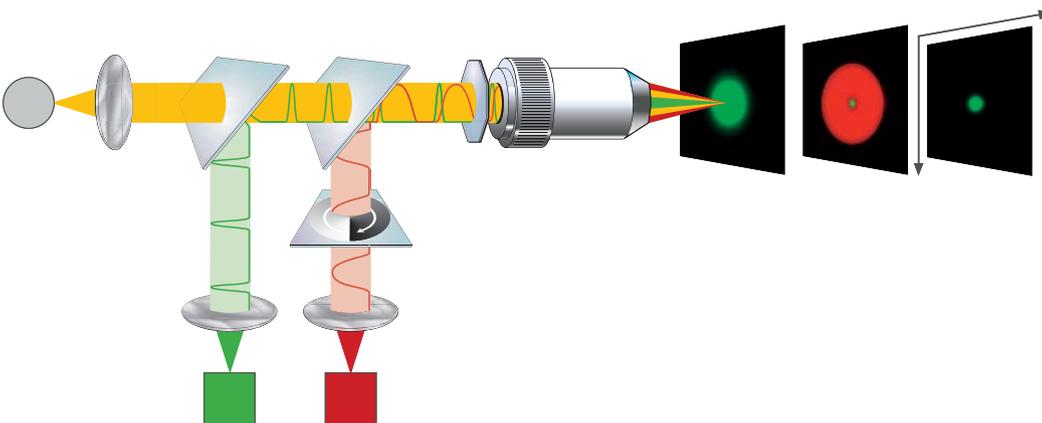


Fig. 1: Simplified excitation and detection light path of a STED microscope. The excitation laser (green) and the STED depletion beam (red) are focused into the specimen to yield a diffraction limited excitation spot and a doughnut shaped STED PSF, respectively. A helical phase plate in the STED beam path generates the doughnut shape of the STED PSF. Fluorescence (yellow) is recorded by a point detector.

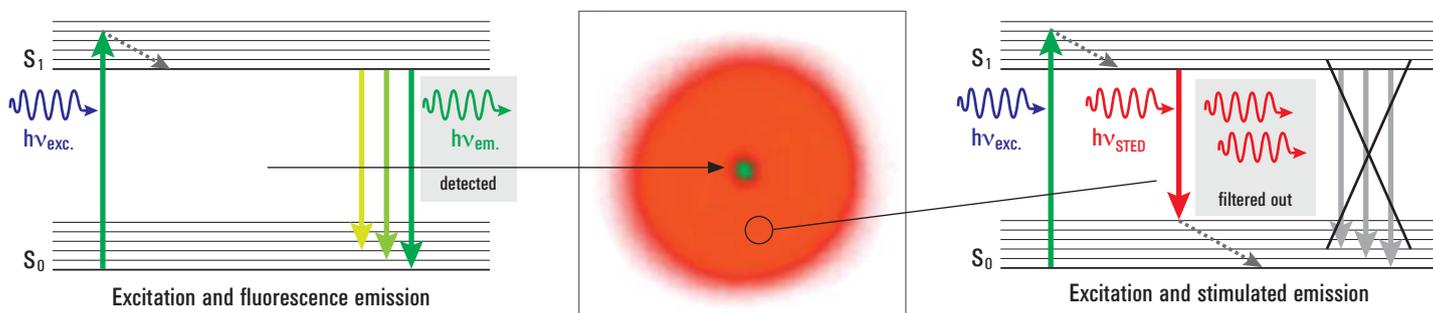


Fig. 2: The involved photophysical processes are confined to different areas of the STED scanning spot. The conventional excitation of the fluorophores that is followed by spontaneous emission of photons with different energies (= wavelengths) dominates inside the ring, where the STED intensity is close to zero (shown on the Jablonski diagram on the left). The STED laser depopulates the excited electronic state S_1 by inducing stimulated emission in the periphery (Jablonski diagram on the right). The released photons are indistinguishable from the STED laser photons and are spectrally filtered out. The process is not related to bleaching and can be repeated many thousands of times.

Dual Color STED – The Principle

The possibility to see biological details far smaller than 100 nm opens a new world for life scientists researching subcellular morphology and dynamics. Multi-color imaging beyond the Abbe limit allows the investigation of how molecules are functionally arranged with respect to one another at the nanoscale.

There are several ways to realize 2C STED microscopy. Leica has chosen a STED implementation that can be referred to as a one-doughnut approach³. It only needs one STED wavelength, two different excitation lines, and the appropriate choice of dye pairs.

In STED microscopy, the fluorophore is the key player (Fig. 3). It is excited by an appropriate laser line and depleted by the STED laser. In order to avoid anti-Stokes excitation generated by the STED beam, which would counteract the resolution increase, the excitation spectrum should be narrow and/or the Stoke shift should

be large. Furthermore, there needs to be a significant amount of emission at the depletion wavelength, as this coincides with the efficiency of stimulated emission. The perfect dye features high brightness and photo stability under the given imaging conditions. Especially in the green/yellow spectral range, many dyes/fluorescent proteins work well with STED microscopy (compare Tab. 1).

For 2C STED, the rules of classic microscopy apply: to acquire images in 2 colors, the best dyes are the spectrally-separated ones. Usually, scientists choose fluorophores that are excited at different wavelengths without cross-excitation and have well separated emission spectra. For STED microscopy, the fluorophores must also have a high cross-section for stimulated emission at the depletion wavelength. The one-doughnut approach uses the same STED line for both dyes and therefore, fluorophores of at least partially overlapping emission must be used. The

Dual Color STED Imaging

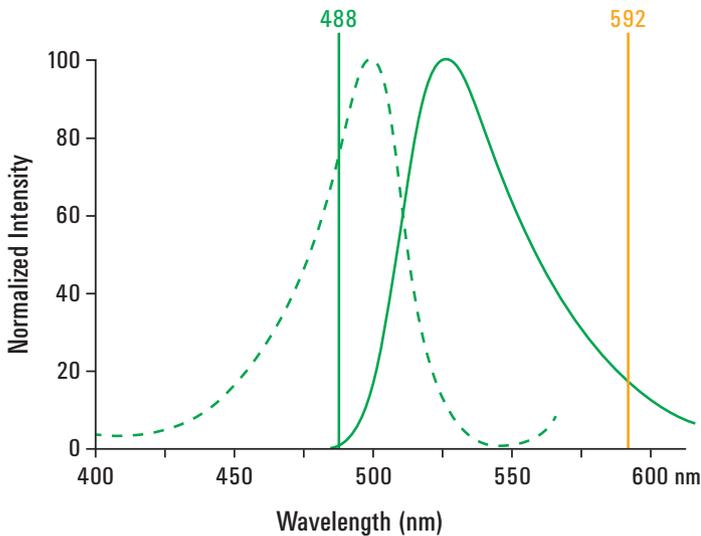


Fig. 3: Oregon Green 488: A typical dye for STED microscopy in the green spectral range. The excitation spectrum is narrow enough to not absorb the depletion beam. There is a high cross-section for stimulated depletion at 592 nm (yellow line) and a good available detection range.

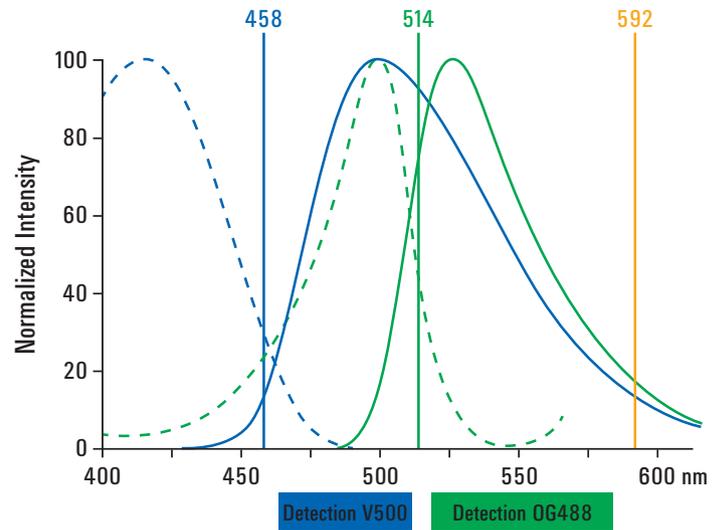


Fig. 4: Example of fluorophores with well separated excitation peaks and partially overlapping emission spectra (BD V500 in blue + Oregon Green 488, displayed in green) suited for 2C STED imaging with the Leica TCS STED CW. In blue, excitation of BD V500 at 458 nm, in green excitation of Oregon Green 488 at 514 nm, in yellow depletion at 592 nm. Please note: Suboptimal excitation does not only help to avoid crosstalk, but also to choose appropriate detection windows.

excitation spectra should be nicely separated in order to achieve good color separation. Combining a classic dye and a dye with a large Stokes shift works best in practice. Fig. 4 shows an

example of a dye pair for the Leica TCS STED CW, which allows super-resolved 2C images to be obtained without crosstalk between channels.



This section describes steps to acquire high quality 2C STED images. As STED is a technology based on confocal microscopy, any sample adapted to confocal is expected to give good results in STED. Tissues as well as cells can be investigated. Any labeling method, immunohistochemistry, immunocytochemistry, FISH, etc., is suitable. The best results are obtained from bright specimens with high contrast. Some tissues with absorbance at the depletion laser wavelength can be problematic such as chloroplasts, pigmentation in zebrafish, or lipofuscin in old brains.

STED microscopy is fully compatible with standard staining and fixation protocols, but fluorophores suited for this super-resolution method need to be applied. Staying as close as possible to the established specimen preparation protocol will give perfect results, by respecting some constraints. This starts with the choice of an appropriate fluorophore combination.

Dye Selection

The Leica TCS STED and TCS STED CW realize STED in two different spectral ranges, but the general principle of dye selection stays the same. The underlying rationale has been explained above and is summarized in Tips & Tricks for dye selection.

Tips & Tricks

Dye Selection for 2C STED

The optimal STED dye features in general:

- Excitability with the available lines
- Some emission at depletion wavelength (correlates with high STED efficiency)
- No anti-Stokes excitation at the depletion wavelength
- High quantum yield
- High photo stability under STED imaging conditions

Dye pairs for 2C STED should further show:

- Little cross-excitation (combination of fluorophores with large and normal Stokes shift)
- Differences in the emission spectra
- Good STED efficiency at the same wavelength

Examples of Dye Combinations

Leica TCS STED CW

- BD Horizon V500 and Oregon Green 488 or Chromeo 505 (best choice, no spectral separation)
- Abberior STAR 440SX and Oregon Green 488 or Chromeo 505 (no spectral separation)
- Pacific orange and Alexa 488 or Oregon Green 488 (dye separation might be necessary)

Leica TCS STED

- Chromeo 494 and ATTO 647N or Abberior Star 635 (best choice, no dye separation necessary)
- Alexa 532 and ATTO 647N or Abberior Star 635 (no dye separation necessary)
- Mega 520 and ATTO 655 or ATTO 665 (dye separation might be necessary)

Leica TCS STED CW

The Leica TCS STED CW is compatible with a variety of common fluorophores such as Alexa 488, FITC, Oregon Green 488, and also genetically encoded markers like eYFP, Venus, and Citrin. In specimens eGFP can yield good results. A dedicated STED objective provides chromatic optimization for excitation and depletion laser lines. Any fluorophore, which can be excited in the range of 458 to 514 nm and shows some emission at 592 nm, is a candidate for STED CW imaging (see Tab. 1 for examples of applicable fluorophores).

Large Stokes shift dyes such as Abberior STAR 440SX, Pacific Orange, and BD Horizon V500 can be combined with standard fluorophores excited at 488 or 514 nm. Choosing appropriate combinations makes channel separation/spectral dye separation obsolete.

Regarding the respective excitation and emission spectra of all dyes tested, the best approach is to employ the spectral differences of BD Horizon V500 and Oregon Green 488/514 (or Chromeo 488/505). By exciting Oregon Green 488 with 514 nm, BD Horizon V500 is not cross-excited. Further, almost no Oregon Green 488 fluorescence falls into the BD Horizon V500 detection window (compare Fig. 4). Images can be acquired without crosstalk or bleed-through, and no post-processing is necessary (Fig. 5).

Fluorophore	Excitation Wavelength (nm)	Provider	Remarks
Standard dyes			
Alexa 488	488 or 514	Invitrogen	
ATTO 488	488 or 514	ATTO-TEC	
Chromeo 488	488 or 514	Active Motif	
Chromeo 505	488 or 514	Active Motif	Recommended for 2C
FITC	488 or 514	Invitrogen	
FluoProbes 488	488 or 514	Interchim	
DyLight 488	488 or 514	Pierce Technology	
Oregon Green 488	488 or 514	Invitrogen	Recommended for 2C
Oregon Green 514	514	Invitrogen	Recommended for 2C
Large Stokes shift dyes			
Abberior STAR 440SX	458	Abberior	Recommended for 2C together with Chromeo 505 or Oregon Green 488
ATTO 425	458	ATTO-TEC	Dye separation required for 2C; resolution not optimal
BD Horizon V500	458	Becton Dickinson	Streptavidin; recommended for 2C together with Chromeo 505 or Oregon Green 488
Cascade yellow	458	Invitrogen	Resolution not optimal
NBD-X	458	Anaspec	No conjugated antibodies available Dye separation required for 2C
Pacific Orange	458	Invitrogen	Dye separation required for 2C
Fluorescent Proteins			
Citrine	514		Recommended
eGFP	488		Results highly dependent on specimen
EmGFP (Emerald Green)	488		
eYFP	514		Recommended
Venus	514		Recommended

Tab 1: Examples of applicable fluorophores for Leica TCS STED CW

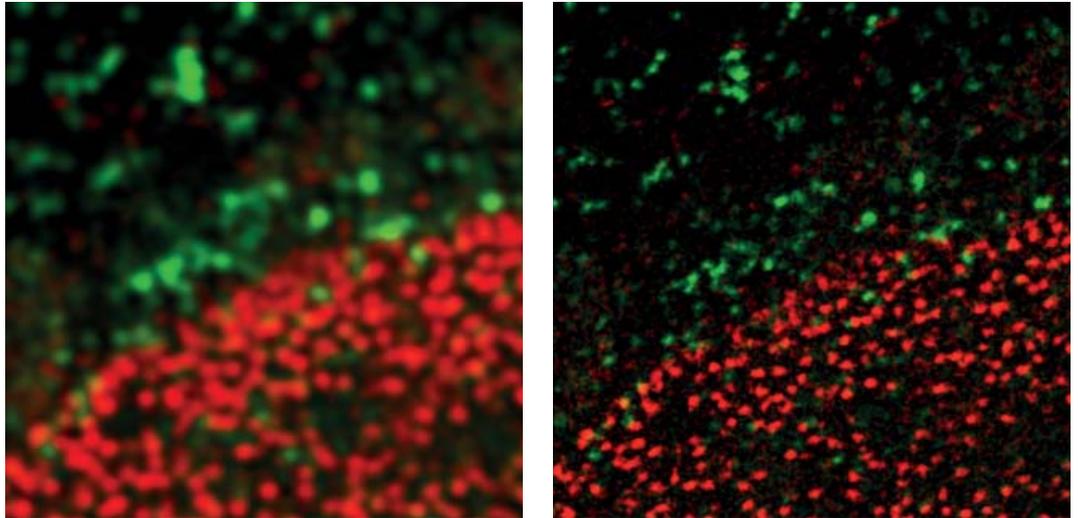


Fig. 5: Nuclear pore complexes and clathrin coated vesicles of adherent cell stained with BD Horizon V500 (red) and Oregon Green 488 (green), respectively.

Tips & Tricks: Specimen Preparation

Typical Immunofluorescence Protocol for Cultured Cells

1. Fix specimen
2. Block non-specific binding sites and permeabilize if necessary, e.g., PBS/normal serum/permeabilizing agent
3. Wash 3x in PBS (optional)
4. Incubate with primary antibodies
5. Wash 3x in PBS
6. Incubate in secondary antibodies. Dilution of secondary antibodies should be tested in order to obtain the best signal-to-noise ratio.
7. Wash 3x in PBS
8. Mount with or without anti-fading reagents.

Note: Use #1.5 coverslips (0.17 mm thick), as microscope objectives are corrected to this thickness.

Leica TCS STED

The Leica TCS STED is based on pulsed lasers for excitation and depletion and works in the deep red (depletion from 740 to 800 nm; excitations at 531 nm and 640 nm). Fewer fluorophores are available for this spectral range. ATTO 647N, ATTO 655, ATTO 665 (ATTO-TEC), and Abberior

Star 635 (Abberior) are the best suited dyes for excitation at 640 nm. They are standard fluorophores with a typical stoke shift. Combining ATTO 647N with a dye having a large stoke shift, e.g., Chromeo 494 (Active Motif) excited at 531 nm, enables super-resolution colocalization studies with the Leica TCS STED (Fig. 6).

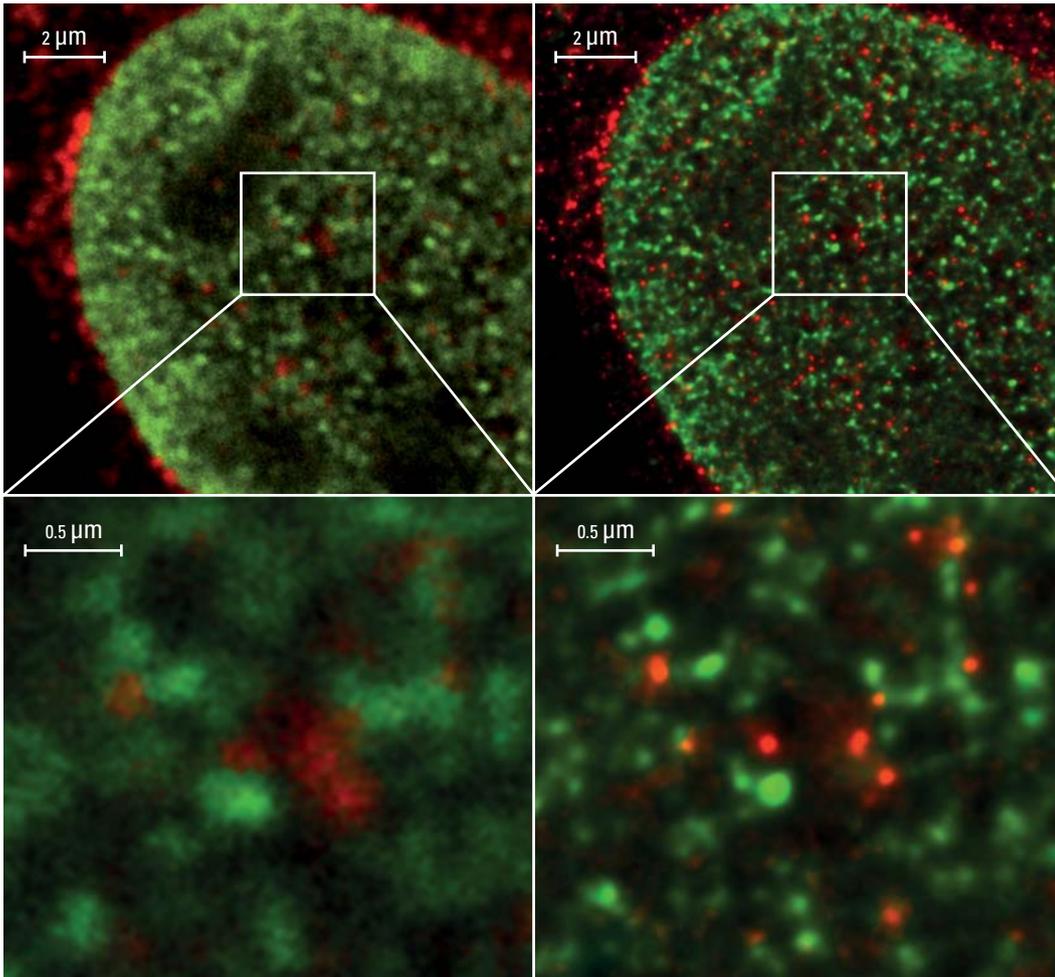


Fig 6: Nuclear structures visualized with Chromeo 494 (green) and ATTO 647N (red). Specimen: courtesy of Dr. L. Schermelleh, LMU Biozentrum, Munich, Germany

Counterstains

Confocal counterstains can provide additional information from STED specimens. Red Alexa or DyLight dyes as well as ATTO 543, 565, 594 for example, can be used for Leica TCS STED CW specimens.

For the Leica TCS STED, green dyes such as ATTO 488 can be applied to investigate a third structure in the specimen at confocal resolution.

Many scientists are interested in seeing the nuclei in their specimens. When imaging with the

Leica TCS STED, SYTOX blue (Invitrogen) could be added to the specimen to this end (excitation at 458 nm).

For the Leica TCS STED CW, nuclear labels such as Draq5™ (Biostatus Limited), or infrared dyes (TOTO, YOYO and ToPro, Invitrogen), should be used. Neither SYTOX blue nor DAPI/Hoechst should be applied for Leica TCS STED CW, as in some cases they are excited by the 592 nm depletion beam and cause a diffuse background.

Mounting

In microscopy, changes in the refractive index along the optical path, including the specimen itself, act like an optical element leading to a deformation of the PSF and a decrease in resolution. In STED microscopy, as excitation and depletion PSF are involved, a mismatch has a higher impact than in conventional microscopy. In order to obtain the best resolution, the Leica TCS STED and TCS STED CW are equipped with special 100x 1.4 NA oil objectives which are corrected for coverslips of 0.170 mm thickness. Oil (Leica Type F Immersion Fluid, $n_e^{23} = 1.518$) is used as immersion medium. Therefore, the refractive index of the mounting medium should be as close as possible to 1.518, especially when

high penetration depth is desired. Staying close to the cover glass, e. g., within 10–20 μm imaging in aqueous solution, can be done without problems. Most embedding media work (see Tab. 2 for a few examples and the appendix for the protocols).

Thiodiethanol (TDE, Sigma, #88559) has been used with excellent results, especially for deep tissue imaging. The TDE concentration must be gradually enhanced to obtain a final refractive index of 1.514, which is reached using a TDE concentration of 97%. If using TDE, the coverslip must be sealed with, for example, slow drying transparent nail polish, white glue or any sealant

Mounting media	RI	Remarks
TDE	1.514	
ProLong Gold	1.46	Hardening, variation in batch quality
Mowiol +/- 2.5% DABCO	1.5	Hardening
86% glycerol + 4% NPG*	1.452	
86% glycerol + 2.5% DABCO**	1.452	

Tab 2: Some examples of mounting media for STED microscopy

* N-propyl-gallate

** 1,4-Diazabicyclo-8,2,2,2-octan;
Fluka #33480

devoid of autofluorescence. Please note that TDE can effect the quantum yield of dyes. For a detailed protocol and further information please refer to Staudt et. al⁴.

Most commercial mounting media are also compatible. For example, Mowiol 4-88, Calbiochem #475904 (see protocol in appendix), has always given good results. ProLong Gold antifade reagent (Invitrogen, #P36930 or #P36934) has been applied with success. This mounting medium increases its refractive index over time up to 1.46. Still, some batches of ProLong Gold show strange behavior and have a highly orange-colored background.

Homemade mounting media are also suitable. A solution with 86% glycerol in PBS added with anti-fading works fine, especially for cells attached to the coverslip or to image thin specimens (see protocol in appendix).

There are few mounting media not compatible with STED microscopy due to various reasons (absorption of depletion beam, quenching of large Stokes shift dyes, etc.). This is the case for Vectashield (Vectorlabs), and SlowFade (Invitrogen). Also Paraphenylenediamine (PPD), an anti-fading reagent, was reported to give an orange background absorbing the 592 nm depletion wavelength.

Image Acquisition

Both Leica STED systems are based on the Leica TCS SP5 confocal microscope and are easily controlled via LAS AF (Leica Application Suite Advanced Fluorescence) software. For 2C STED, sequential color acquisitions are recommended for optimal results (see Tips & Tricks: Sequential scanning).

With the Leica TCS STED CW, detection can be performed on the spectral detection unit using either Photomultiplier Tubes (PMTs) or Hybrid Detectors (HyD). The latter fit particularly well STED CW imaging due the high sensitivity, low background and high quantum yield in the green/yellow range. The combination of resonant scanner and HyD is highly attractive for Leica TCS STED CW imaging, as fast scanning enhances photo stability, especially under STED conditions and increases cell viability in the case of live cell

nanoscopy. External detection using avalanche photodiodes (APD) via the X1 port is an additional option. As the Leica TCS STED operates in the far red, APDs equipped with a special filter cube are still the first choice due to their outstanding sensitivity in this spectral range. HyDs cannot be applied for STED imaging with the Leica TCS STED.

Tables 3 and 4 give examples of acquisition settings for 2C images with the Leica TCS STED CW and Leica TCS STED.

Leica TCS STED CW					
Dye1			Dye2		
Name	Excitation (nm)	Emission (nm)	Name	Excitation (nm)	Emission (nm)
BD Horizon V500	458	460–510	Oregon Green 488 or 514	514	515–580
Abberior STAR 440SX	458	465–515	Oregon Green 488/Chromeo 505	514	515–580
Pacific Orange	458	535–580	Alexa 488 (or any green dye)	488	500–580
NBD-X	458	500–580	Alexa 488 (or any green dye)	488	500–580

Tab 3: Examples of settings for 2C STED CW acquisitions

Leica TCS STED					
Dye1			Dye2		
Name	Excitation (nm)	Emission (nm)	Name	Excitation (nm)	Emission (nm)
Depletion at 745–765 nm					
Chromeo 494	531	550–605	ATTO 647N or Abberior Star 635	640	665–705
Alexa 532	531	550–605	ATTO 647N or Abberior Star 635	640	665–705
Depletion at 770–785 nm					
Mega 520	531	665–705	ATTO 655 or ATTO 665	640	665–705

Tab 4: Examples of settings for 2C STED acquisitions

Tips & Tricks

Sequential Acquisition

In order to avoid crosstalk, acquisition should be done in sequential mode, meaning that lasers and detectors are alternatively (not simultaneously) switched on to record to the different channels. The user has a choice among 3 different sequential modes, which define how the different channels are acquired:

- Between lines
- Between frames
- Between stacks

2C Image Acquisition with the Leica TCS STED CW

- Use fast scanning to increase photostability
- Use sequential scanning to reduce crosstalk
- Use line sequential scanning for drifting/moving specimen
- Consider suboptimal excitation to reduce crosstalk and give more room to adjust the spectral detectors
- Use the spectral detector to reduce crosstalk and/or maximize signal

2C Image Acquisition with the Leica TCS STED

- Tune the depletion laser to the lowest wavelength applicable without yielding disturbing anti-Stokes excitation
- Use scan speeds > 100 Hz and averaging/accumulation for best results
- Use line sequential scanning for drifting/moving specimen

Image Processing

For a specimen labeled with a green dye such as Alexa 488 and a dye such as NBD-X or Pacific Orange, it may be necessary to prepare single labels with the individual dyes in order to perform dye separation by post processing after

acquisition. Described in 2005⁵, spectral unmixing is a method to separate overlapping fluorescences in a quantitative way. This method is fully implemented in LAS AF under Tools/Dye Separation/Channel (see result in Fig. 7).

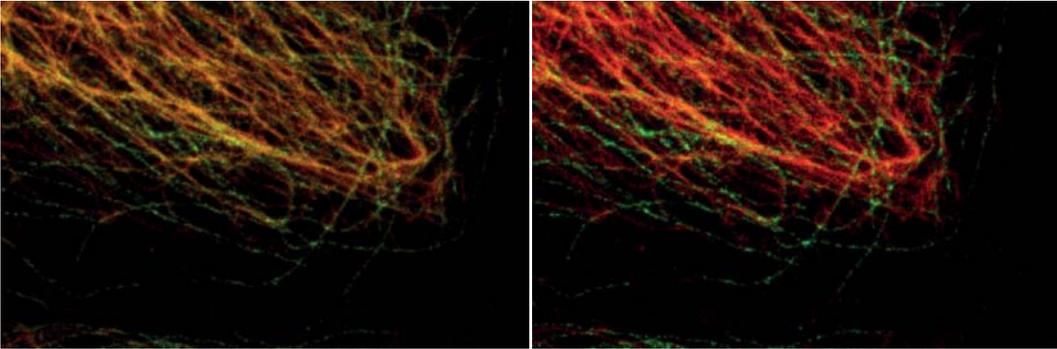


Fig. 7: Images before and after spectral separation. On the left: raw image of the double label (NBD-X in red and Alexa 488 in green) with crosstalk. On the right: spectrally separated image.

For specimen labeled with spectrally better separated fluorophores, like BD Horizon V500 and Oregon Green 505 or Chromeo 494 and ATTO 647N, no post processing dye separation is necessary.

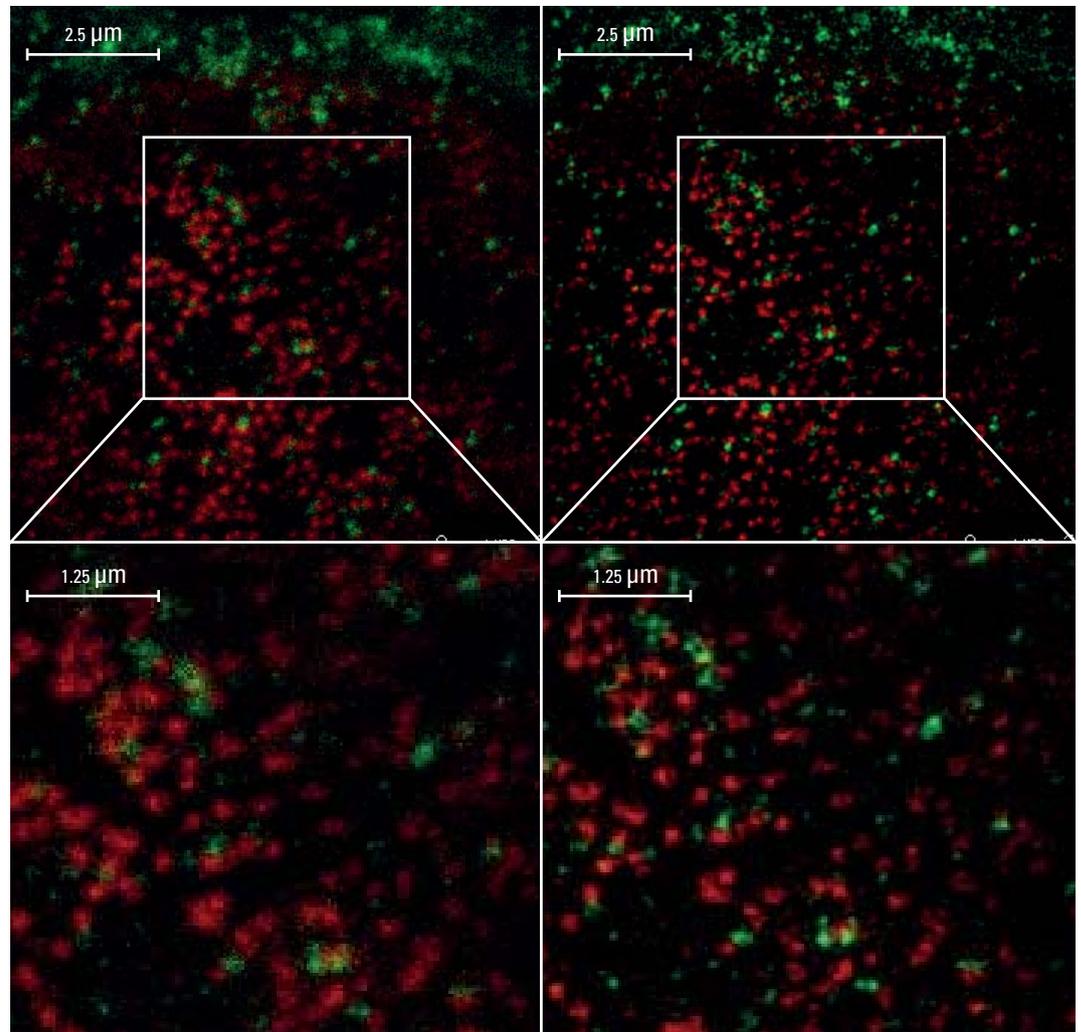


Fig. 8: 2C STED CW. Left: raw STED data. Right: deconvolved STED images. Specimen: courtesy of Rebecca Medda, Max Planck Institute for Biophysical Chemistry, Goettingen, Germany.

In STED microscopy, the PSF follows a Lorentz distribution. Therefore, deconvolution can also be applied to improve STED images. Fig. 8 illustrates a 2C STED image before and after deconvolution.

Deconvolution algorithms suited for STED are implemented in LAS AF.

Please note that deconvolution is a drastic post processing step. To avoid artifacts, it should be carefully used, and the results should always be confirmed by comparing them with the original data. This applies for STED data as well as for work with all other microscopic images.

Conclusions

STED microscopy is a pure optical technology. It is based on confocal principles. All rules applying to good confocal imaging and specimen preparation also apply to STED imaging. The best results will be obtained from specimens with excellent signal-to-noise ratio. Therefore, the labeling steps are crucial, and a good combination of fluorophores simplifies image acquisition and avoids postprocessing.

Appendix: Possible mounting media

Mowiol embedding media preparation protocol:

- Start with 6 g Glycerol (analytical grade)
- Add 2.4 g Mowiol 4-88 (Calbiochem #475904)
- Add 6 ml Aqua dest.
- Add 12 ml 0.2 M TRIS buffer pH 8
- Add 2.5% DABCO (=Anti-Bleaching-Reagent 1.4-Diazabicyclo-82.2.29-octan; Fluka #33480)
- Stir for 4 hours (magnetic stirrer)
- Let suspension rest for 2 hours
- Incubate for 10 min at 50 °C (water bath)
- Centrifuge at 5000x g for 15 min
- Take the supernatant and freeze it in aliquots at -20 °C

Glycerol – PBS-antifading:

- 86% glycerol
- 4% n-propyl-gallate (NPG) or 2.5% DABCO

Thiodiethanol

- TDE, Sigma, #88559) has been used with excellent results. "2,2'-thiodiethanol: a new water soluble mounting medium for high resolution optical microscopy." *Microsc Res Tech.* 70(1):1-9. The TDE concentration must be gradually enhanced to obtain a final refractive index of 1.514, which is reached using a TDE concentration of 97%. For further details see T. Staudt et al.⁴.
- If using TDE, the coverslip must be sealed using transparent nail polish.

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